Strategies for Imaging Priority Targets:
A workshop regarding what in-vivo molecular imaging probes are needed to support future translational studies in cancer therapeutics

Frankfurt, Germany
November 18, 2002

Introduction

Most Important Objective
The challenge is to measure an early effect that is predictive of objective response.

The recent explosion in genetic information, a result of the sequencing of the human genome and evolving genomic and proteomic technologies, has lead to the identification of a plethora of genes that are involved in the development and/or progression of many diseases. With respect to oncology, these technologies, coupled with advances made in the field of *in vivo* imaging during the past 25 years, have created the opportunity to non-invasively image cancer at the molecular level.

Molecular imaging can be a powerful tool when applied throughout clinical drug development, from identifying clinically relevant mechanisms through monitoring response in patients. Strategies for Imaging Priority Targets, a one-day workshop, brought together an international group of biologists, chemists and imaging scientists for the purpose of developing a consensus on which molecular targets would best serve as the next imaging probes to support future translational studies in cancer therapeutics.

General Comments Applicable to All Target Areas

Collaborations
It was stated repeatedly throughout the workshop that key connections must be established between the therapeutic development scientists and imaging scientists. One of the major issues that imaging researchers currently face is late arrival into the drug development process. Most identify imaging targets by searching the literature, and as a result the target is already four to five years old. At this late stage, a useful imaging agent may be developed, however, it may be too late to impact early drug development or clinical decision-making. On the other hand, there is also a downside for getting involved too early in the process, as the drug or target may never make it into the clinic. ***What is the best way to balance this?***

Use of microarrays to identify potential imaging targets
Gene arrays may be useful in the selection of potential imaging targets. For example, microarray analysis can potentially identify thousands of targets that distinguish between tumor types.

Benefit of developing imaging agents early in drug development process
It may be beneficial to label a drug early in the drug development process, pre-Phase I, to determine if the drug is actually getting to the tumor. It was suggested that imaging scientists should be involved five years before the drug enters Phase I trials. An example was provided by one of the participants where a drug was labeled pre-Phase I, and they found that it wasn't getting into some tumors, but it got into others. In this scenario, tumor types could more confidently be chosen prior to clinical trials. At the same time, the technical issues can be addressed, and the drug can be re-designed. The take home message: There is value in knowing tumor uptake.

Epithelial cancer models
Most of the epithelial cancers are not like CML or GIST. Instead, they contain multiple genetic abnormalities that drive the disease. Thus, Gleevec may not be a good model of targeted drugs for epithelial tumors; the mixed clinical experience with Iressa may be closer to reality.

Benefit of imaging agents in clinical trials
Although excellent data have been generated from the invasive biopsy approach, it is undesirable in clinical trials. It slows down Phase I clinical studies, and since all of the patients must have biopsies taken, not every patient is suitable. In addition, there are ethical and practical issues associated with invasive approaches. Repeat biopsies are clearly not going to be possible or sensible, as these studies progress in Phase II/III trials. This is where the imaging technologies could really be useful.

Receptor Tyrosine Kinases (RTK)

Top RTK's Identifed:
- Epithelial Growth Factor Receptor (EGFR)
- ErbB2
- Vascular Endothelial Growth Factor Receptor (VEGFR)
- Platelet-derived Growth Factor Receptor (PDGFR)

Moderators
Biology- Julie Cherrington, SUGEN, Inc.
Imaging- Henry Van Brocklin, Lawrence Berkeley National Laboratory

Background

The ability to monitor the inhibition of receptor tyrosine kinase phosphorylation has traditionally been through the use of immunohistochemistry and Western blot analysis. The limitation of these techniques, however, is the availability of biopsies of many tumor types. Molecular imaging could allow one to monitor a drug's effect on a tumor at a variety of sites in the pathway. There are two possibilities for RTK imaging agents: those that target events at the cell membrane, and those that target an effect of the phosphorylation process upstream vs. downstream once it has occurred, such as hypoxia or apoptosis.

The VEGFR, KIT and MET are three widely investigated receptor tyrosine kinases.

VEGFR
VEGF, also know as vascular permeability factor, plays a key role in tumorigenesis via signaling through VEGFR1 (FLT-1) and VEGFR2 (KDR) and is central to tumor angiogenesis. The downstream effects include: vascular permeability, proliferation, migration, invasion and survival.

Both small molecule VEGFR inhibitors and antibodies against VEGF are being used, both in the pre-clinical and clinical setting, and have demonstrated inhibition of tumor blood flow and vascular permeability. Magnetic resonance imaging (MRI) has been used to image the hemodynamic effects.

The small molecule inhibitor, PTK787, is currently being used in two early phase clinical studies. In a Phase I trial of recurrent glioblastoma, a decrease in vascular permeability was observed with Dynamic Contrast Enhanced – Magnetic Resonance Imaging (DCE-MRI) immediately following first treatment. In a patient who achieved a partial response, vascular permeability was decreased compared to baseline.

In a Phase I trial of patients with colorectal cancer liver metastases, patients with stable disease had a significantly greater decrease in Ki at days 2 and 28 than patients with progressive disease as determined using DCE-MRI. Thus, preliminary data suggest that changes in DCE-MRI parameters can be predictive of subsequent biological activity. ***The association of a surrogate endpoint with clinical response is the type of association that is desired, as well as demonstrating a time- and dose-relationship. It would be ideal to be able to use imaging results in Phase I trials to determine optimal biological dosing.***

KIT
The receptor tyrosine kinase KIT plays a key role in autocrine and paracrine tumor cell growth and, like the VEGF receptors, also contains a split kinase domain. Mutations in KIT are very common, particularly in gastrointestinal stromal tumors. The downstream effects include cell survival/continued metabolism and proliferation.

KIT mutations were identified in approximately 90% of patients at baseline in a Gleevec clinical study. In this study, the FDG PET response occurred in one week compared to clinical objective, which took four to six months. Thus, PET imaging provided a very early read out of efficacy.

MET
The MET receptor tyrosine kinase is involved in cell survival, proliferation, morphogenesis, motility and angiogenesis. Hence, blood flow and vascular permeability are targets that can be biological read outs. Bold-MRI has been used for MET/Hepatocyte Growth Factor imaging and is in pre-clinical stages of development.

EGFR
The Epidermal Growth Factor Receptor (EGFR) initiates a very complex signaling pathway in the cell, which is a common attribute for most RTK's. As a result of this commonality, there are a lot of parallels that one can take to direct imaging agents for the various targets. For the EGFR alone, there have been a number of different radiopharmaceuticals and MRI agents targeted against various processes.

A lot of effort has recently been put into the area of tyrosine kinase inhibitors. Is labeling or developing agents out of these inhibitors a viable approach? Will this provide valuable information? Some of the tyrosine kinase inhibitors contain fluorine, which can be substituted by fluorine-18. Work in VanBrocklin and Snyder laboratories have shown that when some drugs, such as Iressa and Tareva, are labeled with fluorine-18 in the para position, the fluorine falls off *in vivo*. Although these drugs can be labeled, to date they have not made very good imaging agents.

Discussion Highlights

Use of surrogates for validation
Can we adequately use surrogate markers that can be obtained kinetically to validate a static biopsy?
Ongoing studies include MRI or PET in the case of RTK inhibitors while performing transcriptional profiling of blood cells in parallel.

Epithelial cell read out
Many years have been spent analyzing blood in an effort to understand the events that are occurring in an epithelial tumor. Many of the signaling pathways that are active in hematopoietic cells, however, are quite different than the signaling pathways that are active in epithelial cells. Since epithelial cells are being targeted, wouldn't it be more appropriate to use an epithelial read out where the drug has to permeate through the blood vessels to the tissue? ***Hair follicles and skin biopsies may be a more appropriate read out for epithelial cells.***

Use of small peptides to measure kinase activity

There are a number of peptide domains that are specific for different kinases. Can enzymes be assayed via substrate peptides? Is this type of approach practical?

Phosphorylation of a small peptide by the kinase of interest may be a potential means of trapping the peptide in the cell. If this peptide is labeled or can be detected in some way, it could serve as a read out of kinase activity. Several groups in the US and UK are currently attempting to use this approach.

Common intermediaries

Common intermediaries may potentially serve as great read outs, such as activation of the mitogen-activated protein kinase (MAPK) pathway, even if these are not the driving events. Effective dosing requirements may potentially be established by measuring the activation or de-activation of specific key pathways. ***The challenge, however, will be to get the molecules across the cell membrane.***

Measuring receptor density versus downstream events

Should receptor density be measured or events that occur downstream of receptor activation? There are conflicting opinions on whether the number of receptor molecules that are present on the cell surface reflect receptor tyrosine kinase activity. Thus, it may be more valuable to query whether the receptor tyrosine kinases that are present on the cell surface, such as EGFR, are actually active.

The conclusion drawn from Iressa pre-clinical data was that EGFR expression did not correlate with clinical results. As a result, EGFR expression was not measured in cancer patients throughout the clinical trials. But what if the pre-clinical xenograft model that was used for Iressa was not a relevant model? What if EGFR expression in the clinic really is important?

Although difficult, a lot of high quality, high information science is conducted in the Phase I setting with invasive methods. ***It was mentioned that perhaps the imaging community should focus on the downstream read outs that are more applicable to larger numbers of patients in Phase II where less intense and less invasive monitoring is highly desired.***

In addition, heterogeneity within a tumor and among tumors is a limiting factor when identifying molecular targets. Thus, this limitation alone necessitates clinically that downstream events like apoptosis and proliferation rates, which are always in common with effective therapies, should be measured.

Competition with endogenous ATP

If a tyrosine kinase inhibitor has been designed to include F-18 for PET imaging, for example, the radionuclide dose will be several logs less than the therapeutic dose of a cold drug. ***Can imaging probes be designed in such a way that would allow them to successfully compete with endogenous ATP?*** A lot of cold drugs have side chains that not only fit in the adenine binding pocket, but also the hydrophobic adjacent pockets which increases the binding affinity. What are the problems that have kept F-18 probes from binding the kinase targets as effectively? What can be done in order to develop these?

Animal models

Animal model systems are going to be one of the biggest challenges in imaging: What is valid, how will they be validated, and what is going to be useful in the patient?

Glucose-related changes

Is a glucose-related change a useful read out of tyrosine kinase inhibitor effects? Changes in FDG-uptake appear to correlate closely with subsequent objective response of GIST tumors to imatinib (Demetri et al. N Engl J Med (2002) 347:472-480). Although it will depend on tumor type, how useful PET is going to be across solid tumors in predicting response will remain to be seen.

Questions/Challenges Addressed

- What is an appropriate target that will change in response to therapy? Should receptor tyrosine kinases be targeted? If these are present on all cells, such as EGFR, then the therapeutic index becomes a limitation.

- Is it important to measure receptor density?

- Should receptor binding or downstream events be measured?

- Should specific or generic signaling pathways be measured? Or both?

- The presence of receptor signaling pathways or the receptors themselves does not necessarily guarantee the success of the clinical agent as shown by work conducted on EGFR tyrosine kinase inhibitors. Iressa is one example of this.

- As molecularly targeted therapies are applied to heterogenous human tumors, beneficial drugs may be lost due to unrealistic expectations. Are useful drugs going to be missed because of the demands made on showing single agent responses or in having some degree of predictive power in selecting patients?

- How useful are animal model systems for predicting drug efficacy and safety in humans?

- What is considered a valid pre-clinical animal model? With respect to the Iressa trial, the animal model system used was not predictive.

- How do we address cancers for which animal model systems do not exist? For example, there is currently not one animal model that can effectively mimic non-small cell lung cancer.

- How much does blood flow affect the measurement of imaging agents?

- Is measuring phosphorylation or changes in ATP level relevant with respect to the development of RTK imaging agents?

- The issue of clinical response is important because some of the kinase inhibitors may prolong disease progression, and measuring progression-free survival is hard to measure anatomically and clinically.

Intracellular Signaling Kinases

<u>Moderators</u>
Biology- Judith Sebolt-Leopold, Pfizer
Imaging- Derek MacLean, PetNet, LA Tech Center

> A consensus could not be reached on top intracellular signaling kinases as potential imaging targets.

Background

There are three levels of biomarkers that are currently used for kinase drug development programs. These are:

- Level 1: Biochemical modulation of target
  Examples include phosphorylation of ERK (MEK and Raf kinase targets), Forkhead (AKT), 4E-BPl (mTOR), pRB (cyclin-dependent kinases)

  Advantages
  - Provides a link between mechanism of action and activity
  - Early pharmacodynamic testing in pre-clinical models allows for selection of optimal compound for development
  - Pre-clinical testing can predict threshold plasma levels required for clinical activity
  - Increase confidence of go/no go decision on the basis of Phase I data

- Level 2: Markers of physiological response (provides functional modulation of response such as proliferation, apoptosis, tumor blood flow)
  Examples include Ki67, caspase activation, PARP cleavage. The latter two occur due to proteolytic activity so could be measured using probes described below in proteasome/protease section.

  Advantages
  - Provides functional evidence that target inhibition affects the tumor
  - Can remedy the lack of a useful Level 1 biomarker

- Level 3: Surrogate markers of efficacy
  Examples include M-protein, PSA, CEA, CA l25, tumor regression

Some of the current pitfalls associated with biomarker assays include:

- Ability to observe target modulation may not always be straightforward. For example, some targets may be hampered by low levels of endogenous phosphorylation.
- Difficulty in obtaining sufficient amounts of clinical tumor biopsies
- Tumor heterogeneity and sampling variability
- Choice and analysis of surrogate tissues is fraught with limitations
- Quantitation by immunohistochemistry can be cumbersome
- What degree of target inhibition is required to see meaningful clinical activity? Are pre-clinical models predictive? Clinical biology may be different from the best of our models.

What is an appropriate target for intracellular kinases?

- pTyr peptide?
- PolyPro peptide?
- ATP; substrate peptide?
- Phospholipid?

Discussion Highlights

Challenges of targeting intracellular pathways

The complex oncogenic pathways are extremely daunting from a chemistry perspective. In addition, the field of clinical oncology is plagued by examples of negative clinical trials where it is not clear if lack of efficacy was due to the inhibitor target being inconsequential or whether the requisite degree of target inhibition was not achieved.

ATP competitive compounds
All of the compounds currently in development are ATP competitive (ATP binding site compounds). Substrate peptides have been quite disappointing in terms of specificity. A striking observation is how many diverse kinases are inhibited by similar compounds, often not as predicted based on sequence homology.

With respect to drug efficacy, the drug needs to compete with nanomolar ATP. With imaging, however, how potent does the inhibitor need to be in order to realistically see accumulation at the site of the molecular target?

Intracellular kinase targeting
It is going to be difficult to target intracellular kinases because the drug or imaging agent will need to get through the cell membrane.

Imaging strategies identified
Imaging strategies identified were 1) labeling a drug, and 2) labeling a substrate for an enzyme. The latter may have specificity problems (discussed above), and the imaging community may need to develop analogues that are both good substrates for the enzyme and provide good imaging characteristics. Concern was expressed that it may be too much hassle to develop such good imaging agents. There was a suggestion that technology was being developed (e.g. Uppsala) to use high throughput screening to discover imaging ligands.

Downstream convergent points
Should downstream convergent points be targeted as potential imaging agents? For example, not a single signal transduction inhibitor has been identified that does not affect either the PI3K or MAPK pathways. This may be a great read out for determining drug dosage. ***It also allows for the development of two or three generic tracers that could potentially be used with multiple different drugs. Thus, the big advantage of using generic tracers is that a different probe will not need to be developed for each drug. May want to consider using both generic and specific probes.***

Diagnostic tests lacking therapeutic utility
One question raised is what is the value of a diagnostic agent that does not have therapeutic utility? If the agent has already been designed to get across the cell membrane, why not design it to also kill the cell? This was met with mixed reviews. It makes business sense to couple a diagnostic with a therapeutic agent. However, tumors are heterogenous, and a number of cell signaling agents would have to be combined. A diagnostic test may be able to characterize the tumor in advance so individualized therapy can be pursued for each patient.

Questions/Challenges Addressed

- How potent do tracer levels need to be?
- Is labeling the substrate of an enzyme useful? A few have tried it but it has proven to be disappointing. Specificity issues.
- Which targets are most dynamic? Over 2000 genes are affected in response to a MEK inhibitor as determined by transcriptional profiling. Do any correlate with shutting down the pathway?
- Is there a useful *in vitro* screening method for identifying what makes a good imaging agent?

Currently in data collection mode.

- Should downstream convergent points be targeted for imaging? May want to use both generic and specific probes.

- Should the precise molecular target read out be an invasive biopsy, ELISA, Western blot or gene array profile and focus the imaging technologies on the generic read outs?

- Use of high-throughput screening for imaging agent development?

- Is it useful to make diagnostic agents that do not have therapeutic utility?

P53 and Related Targets

Top Targets Identified:
- p53
- p53-inducible genes (PIGs)
- Markers of apoptosis
  - Annexin
  - Caspase activity

Moderators
Biology- Albert Fornace, National Cancer Institute
Imaging- David Piwnica-Worms, Washington University

Background

Almost all tumors have alterations in the p53-Mdm2-p19ARF pathway.

Examples of p53 effector genes:
Growth control*: p53, CIP1/WAF1, GADD45, WIP1, IGF-BP3, RB, 14-3-3a, TGF- β2, Inhibin-β, MDM2, EGFR, PCNA, TGFα, ClnG, ClnD1*
Apoptosis: *p53, BAX, FAS1, DR5, seven in absentia, PAG6008, BCL-X, p53AIP1, p53DINP1*
Angiogenesis: *TSP1, BAI1, thrombospondin 2*
Inflammation: *MMP-2 and MMP-9*
Oxidative stress: *p53-inducible gene 1 (PIG1) to PIG14, p85*
DNA repair: *GADD45, DB2 (XPE), CIP1/WAF1, p53R2 PCNA, p53, XPC*
Other: *wig1, GML, Wip1*
Repressed genes: *MAP4, FK506-binding protein 25, BCL2, bFGF, various G2/M proteins, etc.*

There are multiple ways to image p53-mediated effects. These include:
- Measuring transcriptional activation. Small-molecules could be developed to measure protein-DNA or protein-protein interactions
- Developing a small molecule that binds to mutant p53
- Measuring post-translational function (p53 targeted promoters)
- Measuring downstream targets such as ubiquitin-mediated degradation or acetylation
- Imaging transcriptional regulation of p53-dependent genes with PET *in vivo*.
- Noninvasive bioluminescence imaging of 26S proteasome function (also see proteasome pathway discussion).

Discussion Highlights

Target validation of p53-inducible genes
p53-inducible genes do not necessarily correlate tightly with p53 status, especially when a tumor is not under stress. Without stress, many of these genes have other regulatory functions. Thus, when considering the use of p53-inducible genes as potential imaging targets, target validation will be essential.

Use of oligonucleotides as imaging agents
Currently, the presentation of oligonucleotides (oligo's) to their appropriate target is a problem as only approximately 0.1% have reached their target to date. The other 99.9% represent background noise. As long as the oligo is non-toxic, then it can be considered an avenue for therapeutics, however, this is not the case for imaging. What is considered a good drug and what is considered a good imaging agent may be completely different with respect to target to background ratio.

Two important issues that need to be addressed: 1) delivery and 2) non-specific binding.

The use of oligo's as imaging agents is considered a difficult area although it is actively being pursued in Europe. There has been some development by a Swedish group who are combining oligos with other molecules to provide amplification and increased delivery to the target.

Amplification
What is considered a good imaging target is a molecule that has been amplified. ***It was mentioned that perhaps amplification techniques could be pursued for mRNA.***

Protein-protein interactions
Small molecule imaging agents could be designed to specifically serve as a read out for protein-protein interactions. Although this could be achieved by using either a reporter gene strategy or small molecule, the small molecule approach is considered more attractive as it could be used in man. However, this is considered a high-risk area.

Reporter gene strategy
It was mentioned that although a reporter gene strategy is appropriate for animals and has matured well, this type of strategy would probably be considered inappropriate in man, except in the gene therapy setting. ***Development of reporter assays is not needed for drug development. Instead, what is needed is something that can be used further into development, such as an intrinsic prognostic marker that is predictive of response. This marker will have to change in response to the pathway being activated.***

Decoy development
An oligo can be developed that contains a hairpin-like structure, creating a mock site for transcription factor binding. Instead of binding genomic DNA, the transcription factor will bind to the bait, thus blocking transcription. An amplification mechanism can be built into the decoy approach, however, this has yet to be developed in a clinically acceptable way.

Measuring apoptosis
This strategy was discussed in length. There were various approaches identified to measure apoptosis. These include:

- ***Measuring caspase activity***. This approach involves substrate labeling, which is currently being pursued by some groups. (Also see proteasome pathway discussion).

- ***Annexin labeling***. This is currently the most advanced approach. Technetium labeling for SPECT is commercially available. PET imaging of Annexin is available by labeling with ^{124}I or ^{18}F or ^{94}Tc. The advantage of ^{124}I is that it can be measured over several days, but the disadvantage is that not all of the emissions are photons. Ideally, ^{18}F would be used for labeling. Some groups are currently exploring this avenue, however, this work has not reached a level of *in vivo* validation.

Note: If the Annexin V system is to be selective for imaging apoptosis, a measure of the integrity of the plasma membrane of the target cells would be needed. In the laboratory, propidium iodide staining is one established approach - the lack of nuclear staining documents the intactness of the plasma membrane. In man, this could perhaps be achieved by 125I-albumin or 111In-DTPA labeling as a measure of volume of distribution with compounds expected to remain in the extracellular spaces to provide an assessment of the level of intactness of the cells in the tumor. In the absence of a second label, Annexin V does not distinguish between apoptosis (membrane intact) and necrosis (membrane disrupted).

- MRI technologies using Annexin-like molecules are in development. MRI has the advantage of being able to measure dynamic flow at the same time. It is uncertain as to how far this field has developed.

There are some general problems that are associated with measuring apoptosis:

(i) The size of the apoptotic signal may only be 3 or 4%.
(ii) The effect of flow on apoptotic measures. Should these measures be normalized to flow?
(iii) There was concern about the transientness of apoptosis.

DNA Methylation

Top Targets Identified:
• Methylated cytosines

Moderators
Biology- Peter Jones, University of Southern California
Imaging- Tom Meade, Northwestern University

Background
There are four base pairs in DNA. However, approximately 4% of all cytosines are methylated by DNA in methyltransferases (DNMTs) after they have been synthesized into DNA. DNMTs read the pattern in the parental strand of DNA and apply methyl groups on the new strand of DNA opposite originally methylated sites. This epigenetic modification permanently suppresses genes, and is formally equivalent to a loss of heterozygosity (LOH) or to a mutation within tumor suppressor gene. There is one important distinction: the gene can be turned back on again with a hypomethylating drug. Thus, this is a highly heritable, but potentia1ly reversible system.

Almost all human cancers have significant methylation changes and important genes, such as tumor suppressors and those involved in apoptosis, are being silenced. These abnormally methylated alleles can be detected in blood or in the lumen of various organs and can be used as a fairly sensitive method for cancer detection.

Discussion Highlights

Imaging goal
The goal is to image a reporter of demethylation.

Use of inhibitors in combination
In order to re-awaken silenced genes, an effective strategy may be to combine a DNA methylation (DNMT) inhibitor with a histone deacetylase (HDAC) inhibitor. There are currently numerous HDAC inhibitors in pre-clinical and clinical trials.

(In) direct imaging of DNA methylation
At the moment, DNA methylation cannot be directly imaged *in vivo*. ***Therefore, indirect methods may need to be sought.***

(i) Combinatorial selection of peptides that will specifically bind to methylation sites. One issue to be addressed is how the imaging probe will cross the nuclear membrane.
(ii) Substrate approaches such as acetate metabolism. However, there was concern that this may be too general of an approach.
(iii) Measurement of acetylated histones.
(iv) Use of caspase as a target to measure DNA methyltransferase.
(v) Measuring cell cycle arrest. Suggestions for this included:
  (a) Measuring senescence.
  (b) DNA proliferation markers such as C thymidine or ^{18}FLT.
  (c) Measuring G_2M arrest.

Ubiquitin Proteasomal Pathway

Top Targets Identified:
- Proteasome
- Matrix metalloproteases
- Cathepsin B
- Caspases
- PARP cleavage
- Transmembrane serine proteases

Moderators
Biology- Bonnie Sloane, Wayne State University
Imaging- Ralph Weissleder, Mass General Hospital

Background

Matrix Metalloprotease I (MMPI) inhibitors in Phase III clinical trials have been unsuccessful. It is not known whether the inhibitors actually reached their target enzyme in the tumors, or if the targets were present in the tumors to begin with. This is one example of how an imaging program could have potentially saved a drug in development.

There are currently 22 clinical trials taking place in the US and Canada that are targeting the proteasome, ***yet no one has come up with a non-invasive way to image this pathway***. The proteasome pathway degrades any molecule as long as it is multi-ubiquinated. ***For imaging***

purposes, the molecules will not only need to cross the plasma membrane to get into the cytoplasm, but they will also need to be able to refold in the cytoplasm so they can be ubiquinated. The best idea that has come up to date is to try to image a receptor on the cell surface that is degraded by the proteasome.
How can proteases be imaged? One method is to label an affinity ligand for a given protease, but this is unlikely to work because of concentration, amplification and delivery issues. The other method is to use activatable imaging probes- these are in one state upon injection and then change their physicochemical properties upon interaction with enzymes.

Protease substrates (activatable):

l) MRI

- Paramagnetic agents, such as gadolinium in a protective "cage" that is susceptible to cleavage by proteases.
- Superparamagnetic agents. Very small, ~20 nm, iron oxide particles that can be constructed to read out, either directly or indirectly, protease signals. When these very small particles are brought into close contact with water, they dramatically change their transfer relaxation time, but not T1 relaxation time. T1-T2 ratio imaging becomes possible when one channel measures the concentration of the substrate, and the other channel is used to measure the activation by a given protease.

2) Near infrared fluorescent agents (NIRF)- Working in the near IR is beneficial because IR travels through tissue very efficiently. How quantitative is this *in vivo*?

- Small molecule. The problem is that a small molecule will get secreted almost immediately.
- Large molecule. There is a need to explore large molecules to exploit longer half lives, more ubiquitous distribution, multi-valency concepts, etc., in an effort to get these sensors to where they need to go. These are based on synthetic graft polymers that have up to 20-30 different reporting groups. One other advantage is that they can be designed to have dual or triple wavelength reporters.
- NIRF agents are the closest with respect to entering clinical trials (e.g., cathepsin B probe, MMP-2 and MMP-9 probes, urokinase plasminogen activator probe).

Discussion Highlights

MMPI trial lessons
What is the global lesson to be learned from the MMPI inhibitor trials? The MMPI trials went directly from Phase I to Phase III. If the trial sponsors had surrogate markers of efficacy and/or toxicity, they could have saved a lot of time and money. Thus, ***pharmacodynamic endpoints are needed***. In addition, patients should be pre-selected based on target expression.

One of the problems with proteases is that not all of the cells within a tumor will express them. It is often the cells within the mass that do not express high levels of proteases, while cells at the invasive edge express high levels.

It is known from studies in plants that if one protease is down-regulated, expression of another may be increased, and often it is not even proteases of the same class that are increased in expression. Since there are ~600 proteases inthe human genome, compensation becomes an issue. In the MMPI trials, just a handful of
proteases were targeted.

Cathepsin B measurement
Experimental evidence suggests that cathepsin B activity may be associated with pre-malignant

colon polyps. Cathepsin B imaging substrates have been developed, the simplest of which is a lysine-lysine that gets cleaved. Although this simple probe may not be specific for cathepsin B,
other probes are under development that are more specific. Nonetheless, the present cathepsin B imaging probe may function as a generic probe for proteolytic activity. Are moregeneric protease probes needed?

Endoscopy-based fluoroscopy techniques, as well as PET approaches are currently being explored.

Cleavage and trapping
PET agents are always radioactiye, thus a cell permeable substrate is needed that, upon cleavage in the cell, loses its permeability and becomes trapped in the cell. Perhaps the permeation domains would get cleaved, for example. There are design issues associated with this strategy and it is currently a work in progress.

Transmembrane serine proteases
Thirty-seven new transmembrane serine proteases have been identified, many of which are highly associated with cancer, and this is considered to be the 'hot' area at the moment. ***Should imaging substrates be designed for these proteases?***

Questions/Challenges Addressed

- Do we want to image a single protease? To date, this is what has been pursued. Probes have been developed to try and distinguish between individual proteases. Perhaps more generic probes are warranted due to compensation, redundancy, cascades, etc.

- Which proteases should be targeted? Should generic probes be designed, perhaps 2-3 probes per class?

- Need to be aware of tumor-stromal/inflammatory cell interactions. Proteases associated with tumors do not
necessarily come from tumor cells; many come from stromal and inflammatory cells.

- Pharmacodynamic endpoints are needed for use in clinical trials.

HSP90 & Related Targets

Top Targets Identified:
- Hsp90 (pharmacodynamic marker)
- Raf proteins
- ErbB2
- Phosphocholine

Moderators
Biology- Paul Workman, ICR
Imaging- Martin Leach, University of London

Background

Hsp90 is a molecular chaperone involved in protein folding and maintaining the stability of a relatively small group of approximately 50 molecules. Hsp90 is overexpressed in many tumors, and is essential for the stability and function of many oncogenic 'client' proteins, including ERBB2, cell cycle kinases, mitotic kinases, and p53. Thus, if Hsp90 is inhibited either genetically or pharmacologically, these client proteins will be degraded as a result of failing to be correctly

folded. In addition, inhibiting Hsp90 will likely block all six hallmark traits of cancer (uncontrolled cell growth, cell signaling, anti-apoptosis, uncontrolled proliferation, angiogenesis and metastasis).

17AAG is a geldanomycin-related Hsp90 inhibitor that is currently showing evidence of clinical activity. This inhibitor is currently in Phase I clinical trials.

Pharmacologica1 audit trail for 17AAG
*= in progress

Active blood/tissue concentrations?	Yes - Plasma - HPLC-UV*
Activity on desired molecular target?	Yes - degradation of client protein* -increases Hsp70* - gene arrays* - proteomics
Modulation of desired biochemical pathway?	Currently in process -inhibits phosphor-Erk* -inhibits phosphor-Akt* -increase/decrease phosphocholine*
Achievement of the desired biological effect?	- Apoptosis, invasion, cell cycle, etc.
Clinical response?	Yes - stable disease*

This audit trail validates the molecular signature of the Hsp90 inhibitor, 17AAG. These responses are not observed with inactive analogs of the active compound, nor with cytotoxic agents or other signaling inhibitors. l7AAG is proving unique.

There has been interest in looking at endogenous signals using MR phosphorus spectroscopy because a number of the pathways affected by 17AAG appear to affect phospholipid metabolism. A Phase I trial is currently being conducted that analyzes patients who are undergoing 17AAG treatment with *in vivo* MR phosphorus spectroscopy.

Discussion Highlights

Of critical importance to drug development
…is for the audit trail to be complete.

Phosphocholine regulation
17AAG treatment results in increased phosphocholine levels; with other Hsp90 inhibitors, phosphocholine production decreases. This is not currently understood.

General versus specific effects
PET glucose-based approaches provide metabolic information, which is an endpoint of a general effect of the drug. The phosphorus approach is more specific to some of the pathways, but not all

that Hsp90 is affecting. Perhaps both approaches could be utilized:

Early stage trial- specific
Late stage trial- general

B-raf

B-raf, a very low expressing kinase, is mutated in ~70% of all melanomas. B-raf is a potential client protein at this stage, albeit a potentially exciting one.

Questions/Challenges Addressed

- What are the critical client proteins that will help to predict response to 17AAG?
- What is the optimal dosing schedule for PhaseII trials? Are there any methodologies that will allow for selecting the best dosing regime?
- How should these agents be used in combination (cytotoxic agents, molecular therapeutics)? Are there predictive markers?
- Can molecular prognostic markers be identified?
- Can molecular markers for resistance be identified?
- Can second generation drugs be improved?

PI3K Pathway

Top imaging targets could not be identified for the PI3K pathway because it is not well enough understood.

Moderators

Biology- Gordon Mills, M.D. Anderson
Imaging- Ken Krohn, University of Washington

Background

The phosphoinositol 3-kinase (PI3K) pathway is mutated in the majority of epithelial tumors (~80%). There are numerous PI3K drugs in development. ***Targets downstream of this pathway could be imaged using MRI approaches.***

Discussion Highlights

PTEN Phosphatase imaging

PTEN in most tumors is lost or inactivated. How does one measure the absence of enzyme? This is unlikely to be a suitable imaging strategy.

AKT phosphory/ation

AKT phosphorylation has been considered for imaging. An antibody approach, such as against phospho-AKT, may be the most appropriate.

Closing Remarks

At this workshop, many more questions were raised than answers could be provided for, but that is the nature of science.

There were some concerns about regulatory requirements. This was particularly relevant where clinical scientific proof of concept experiments were going to be performed versus tightly regulated studies for licensing purposes to develop generic imaging agents. These two developments will need to be considered separately since there are regulatory as well as scientific differences. There were also concerns expressed, particularly from the imaging community, over intellectual property issues with respect to accessing promising molecules for imaging from the pharmaceutical industry.

The biggest take home message is that there is a large gap between drug discovery scientists and the imaging community. In order to move forward, it is critical that interactions be developed between the two groups.

Follow-up meetings were recommended.